Cell

# Lrp5 Controls Bone Formation by Inhibiting Serotonin Synthesis in the Duodenum

Vijay K. Yadav,[1] Je-Hwang Ryu,[1] Nina Suda,[1] Kenji F. Tanaka,[2,10] Jay A. Gingrich,[3] Günther Schütz,[6] Francis H. Glorieux,[7] Cherie Y. Chiang,[8] Jeffrey D. Zajac,[8] Karl L. Insogna,[9] J. John Mann,[4] Rene Hen,[2] Patricia Ducy,[5,*] and Gerard Karsenty[1,*]
[1]Department of Genetics and Development
[2]Departments of Neuroscience, Psychiatry, and Pharmacology
[3]Department of Psychiatry-Developmental Psychobiology
[4]Department of Psychiatry
[5]Department of Pathology
Columbia University, New York, NY 10032, USA
[6]Department of Molecular Biology of the Cell, German Cancer Research Center, D-69120 Heidelberg, Germany
[7]Shriners Hospital for Children, McGill University, Montreal H3G 1A6, QC, Canada
[8]Department of Medicine, University of Melbourne, Heidelberg, VIC 3010, Australia
[9]Department of Internal Medicine, Yale School of Medicine, New Haven, CT 06519, USA
[10]Present address: National Institute for Physiological Sciences, Okazaki 444-8585, Japan
*Correspondence: pd2193@columbia.edu (P.D.), gk2172@columbia.edu (G.K.)
DOI 10.1016/j.cell.2008.09.059

## SUMMARY

Loss- and gain-of-function mutations in the broadly expressed gene *Lrp5* affect bone formation, causing osteoporosis and high bone mass, respectively. Although Lrp5 is viewed as a Wnt coreceptor, osteoblast-specific disruption of *β-Catenin* does not affect bone formation. Instead, we show here that Lrp5 inhibits expression of *Tph1*, the rate-limiting biosynthetic enzyme for serotonin in enterochromaffin cells of the duodenum. Accordingly, decreasing serotonin blood levels normalizes bone formation and bone mass in *Lrp5*-deficient mice, and gut- but not osteoblast-specific *Lrp5* inactivation decreases bone formation in a *β-Catenin*-independent manner. Moreover, gut-specific activation of *Lrp5*, or inactivation of *Tph1*, increases bone mass and prevents ovariectomy-induced bone loss. Serotonin acts on osteoblasts through the Htr1b receptor and CREB to inhibit their proliferation. By identifying duodenum-derived serotonin as a hormone inhibiting bone formation in an Lrp5-dependent manner, this study broadens our understanding of bone remodeling and suggests potential therapies to increase bone mass.

## INTRODUCTION

Bone remodeling, the physiological means whereby vertebrates renew their bones during adulthood, comprises two phases: resorption of preexisting mineralized bone matrix by a specialized cell type, the osteoclast, followed by de novo bone formation by another specialized cell type, the osteoblast. Over the last 15 years, molecular and genetic studies have identified numerous local and systemic regulators of this process and as a result have considerably improved our molecular understanding of both aspects of bone remodeling (Zaidi, 2007).

One of the most intensively studied regulators of bone remodeling is *LDL-receptor related protein 5* (*LRP5*) (Baron and Rawadi, 2007). This interest stems from the fact that *LRP5* loss-of-function mutations cause osteoporosis pseudoglioma (OPPG), a rare disease characterized by severe decreased bone formation and persistence of embryonic eye vascularization leading to low bone mass and blindness (Gong et al., 2001). Other, presumably activating, mutations in *LRP5* cause high bone mass syndrome (Boyden et al., 2002). That different mutations in this gene cause two bone diseases of opposite nature underscores the critical importance in the regulation of bone formation of the pathway(s) controlled by *Lrp5*.

*Lrp5* encodes a broadly expressed cell-surface molecule sharing sequence homology with Arrow, a coreceptor for the growth factor Wingless in *Drosophila* (Bhanot et al., 1996). Based on this sequence homology and, among other evidence, cell-based assays in which Lrp5 and Axin interaction is initiated by Wnt proteins, it is assumed that Lrp5 is a coreceptor for Wnt proteins, the vertebrate homologs of Wingless (Tamai et al., 2004). As a result, OPPG and high bone mass syndrome are viewed as Wnt-related diseases (Krishnan et al., 2006). Three observations, however, challenge this view. First, contrasting with the developmental function of most Wnt proteins, there is no overt skeletal defect in $Lrp5^{-/-}$ embryos. Second, gain-of-function mutations in *Lrp5* do not cause bone tumors as activation of Wnt signaling does in other organs (Moon et al., 2004). Third and more importantly, osteoblast-specific loss- and gain-of-function mutations in *β-Catenin* (*β-Cat*), the molecular node of canonical Wnt signaling, do not affect either bone formation or expression of genes dysregulated upon *Lrp5* inactivation (Glass et al., 2005). Taken individually, none of these observations rules out that Lrp5 functions as a Wnt coreceptor to regulate bone formation. However, when considered together, we

viewed them as an incentive to search for additional/other mechanisms of action of this gene in osteoblasts.

Serotonin is a bioamine generated in brainstem neurons and enterochromaffin cells of the duodenum that does not cross the blood-brain barrier (Mann et al., 1992). Thus, it is de facto a molecule with two functional identities depending on its site of synthesis. Whereas brain-derived serotonin is implicated in cognitive functions (Heath and Hen, 1995), the function(s) of gut-derived serotonin (GDS), which accounts for 95% of total serotonin, is still a matter of debate (Gershon and Tack, 2007). The GDS biosynthetic pathway involves the rate-limiting enzyme tryptophan hydroxylase 1 (Tph1). GDS is released in the general circulation, where most of it is taken up by platelets through a specific transporter (Gershon and Tack, 2007). A small fraction of it, however, remains free in the serum and may conceivably act as a hormone following its binding to serotonin receptors present on target cells (Rand and Reid, 1951). Remarkably, patients taking synthetic serotonin reuptake inhibitors (SSRIs) chronically, a class of drugs increasing extracellular serotonin concentration throughout the body, can have reduced bone mass (Richards et al., 2007).

While searching for molecular mechanisms explaining Lrp5 regulation of bone formation, we identified *Tph1* as the most highly overexpressed gene in *Lrp5*$^{-/-}$ bones. *Tph1* was also increased in *Lrp5*$^{-/-}$ duodenal cells, its primary site of expression. Inhibiting serotonin synthesis in *Lrp5*$^{-/-}$ mice corrects their bone phenotype, and gut- but not osteoblast-specific deletion of *Lrp5* recapitulates the bone phenotype of *Lrp5*$^{-/-}$ mice. The same pathway is affected, in an opposite manner, in the case of *Lrp5* gain-of-function mutation, thus providing a simple molecular basis for the two diseases. Serotonin, after binding to the Htr1b receptor, determines the extent of bone formation by controlling osteoblast proliferation through the regulation of *CyclinD1* expression by CREB. By revealing that *Lrp5* regulates bone mass by inhibiting duodenal synthesis of serotonin, a hormone decreasing bone formation, this study points toward adapted therapies for diseases characterized by an impairment of bone formation.

## RESULTS

### Molecular Signature of *Lrp5* Loss-of-Function Mutation

The different nature of the cellular events leading to a low bone mass phenotype in *Lrp5*$^{-/-}$ (decrease in bone formation) and *β-Cat(ex3)*$_{osb}$$^{-/-}$ (decrease in bone resorption) mice suggested that distinct molecular mechanisms were at work in these two models. Thus, in an effort to elucidate how *Lrp5* regulates bone formation, we looked for a specific molecular signature of *Lrp5* loss-of-function mutation in bone in vivo. For that purpose, we analyzed the expression of genes affecting either cell proliferation (*Cyclins*), osteoblast differentiation (*Runx2*, *Osx*, *Atf4*), bone matrix deposition (*Type I Collagen*), or osteoclast differentiation (*Osteoprotegerin*, *RankL*) in wild-type, *Lrp5*$^{-/-}$, and *β-Cat(ex3)*$_{osb}$$^{-/-}$ bones. The only genes whose expression was decreased in *Lrp5*$^{-/-}$ bones were the regulators of cell proliferation *CycD1*, *D2*, and *E1* (Figure 1A). Consistent with the fact that osteoblast number is normal in *β-Cat(ex3)*$_{osb}$$^{-/-}$ mice, expression of the *Cyclin* genes was not affected in *β-Cat(ex3)*$_{osb}$$^{-/-}$ bones; conversely, genes whose expression was affected in *β-Cat(ex3)*$_{osb}$$^{-/-}$ bones were normally expressed in *Lrp5*$^{-/-}$ bones (Figure 1A).

In striking contrast to the paucity of osteoblasts present in *Lrp5*$^{-/-}$ bones (Kato et al., 2002), *Lrp5*$^{-/-}$ osteoblasts proliferated as well as WT cells ex vivo (Figure 1B). We interpreted the discrepancy between the in vivo and ex vivo proliferation abilities of the *Lrp5*$^{-/-}$ osteoblasts as indicating that *Lrp5* loss-of-function mutations affect osteoblast proliferation through extracellular signals that may not originate from osteoblasts, in other words that *Lrp5*-related bone diseases may not originate from bones.

As a first attempt to test the aforementioned hypothesis, we reanalyzed results of a microarray experiment performed earlier (Glass et al., 2005). The gene most highly expressed in *Lrp5*$^{-/-}$ compared to WT bones was *Tph1*, which encodes an enzyme initiating synthesis outside the brain of serotonin, a secreted molecule that may affect bone mass (Warden et al., 2005) (Figure 1C). Real-time PCR analysis verified that *Tph1* was overexpressed in *Lrp5*$^{-/-}$ osteoblasts and bones (Figure 1D). *Tph1* is predominantly expressed in duodenum in WT mice and it was also vastly overexpressed in this organ in *Lrp5*$^{-/-}$ mice, where its expression was 1500-fold higher than in osteoblasts and 150-fold higher than in any other part of the gastrointestinal tract (Figures 1E and 1F). *Tph1* expression was not affected in *β-Cat(ex3)*$_{osb}$$^{-/-}$ bones, further differentiating Lrp5 and canonical Wnt signaling regulations of bone mass (see Figure S1A available online). In contrast, neither expression of *Tph2*, the enzyme initiating serotonin synthesis in the brain, nor brain serotonin content was affected in *Lrp5*$^{-/-}$ mice (Figures S1B and S1C). This latter observation is consistent with the notion that serotonin does not cross the blood-brain barrier (Mann et al., 1992).

Multiple correlative evidence suggested that this increase in *Tph1* expression is implicated in the development of the *Lrp5*$^{-/-}$ mice bone phenotype. First, *Tph1* expression was normal in newborn *Lrp5*$^{-/-}$ mice that do not display overt bone abnormalities but increased steadily thereafter (Figures 1G, S1D, and S1E). A significant increase in *Tph1* expression was first detected at 2 weeks of age, that is, 2 weeks before the low bone mass phenotype becomes apparent in *Lrp5*$^{-/-}$ mice. Consistent with this increase in *Tph1* expression, blood serotonin levels increased as a function of time in *Lrp5*$^{-/-}$ mice (Figure 1H). This large increase in blood serotonin levels was not due to more platelets in *Lrp5*$^{-/-}$ mice (Figure S1F). Second, *Tph1* expression and blood serotonin levels were increased in *Lrp5*$^{+/-}$ mice that also have a low bone mass (Figures 1G and 1H). Third, there was in three OPPG patients tested (Gong et al., 2001; Toomes et al., 2004) a 4- to 5-fold increase in circulating serotonin levels when compared to age-matched controls; moreover, the heterozygous parent of one of these patients also had higher circulating serotonin levels than control individuals (Figures 1I and S1G). Thus, Lrp5 regulates blood serotonin level in both mice and humans.

### Increased Circulating Serotonin Level as a Determinant of the *Lrp5*$^{-/-}$ Mice Bone Phenotype

To determine whether the increase in circulating serotonin level induced by the increase in *Tph1* expression is a cause of the

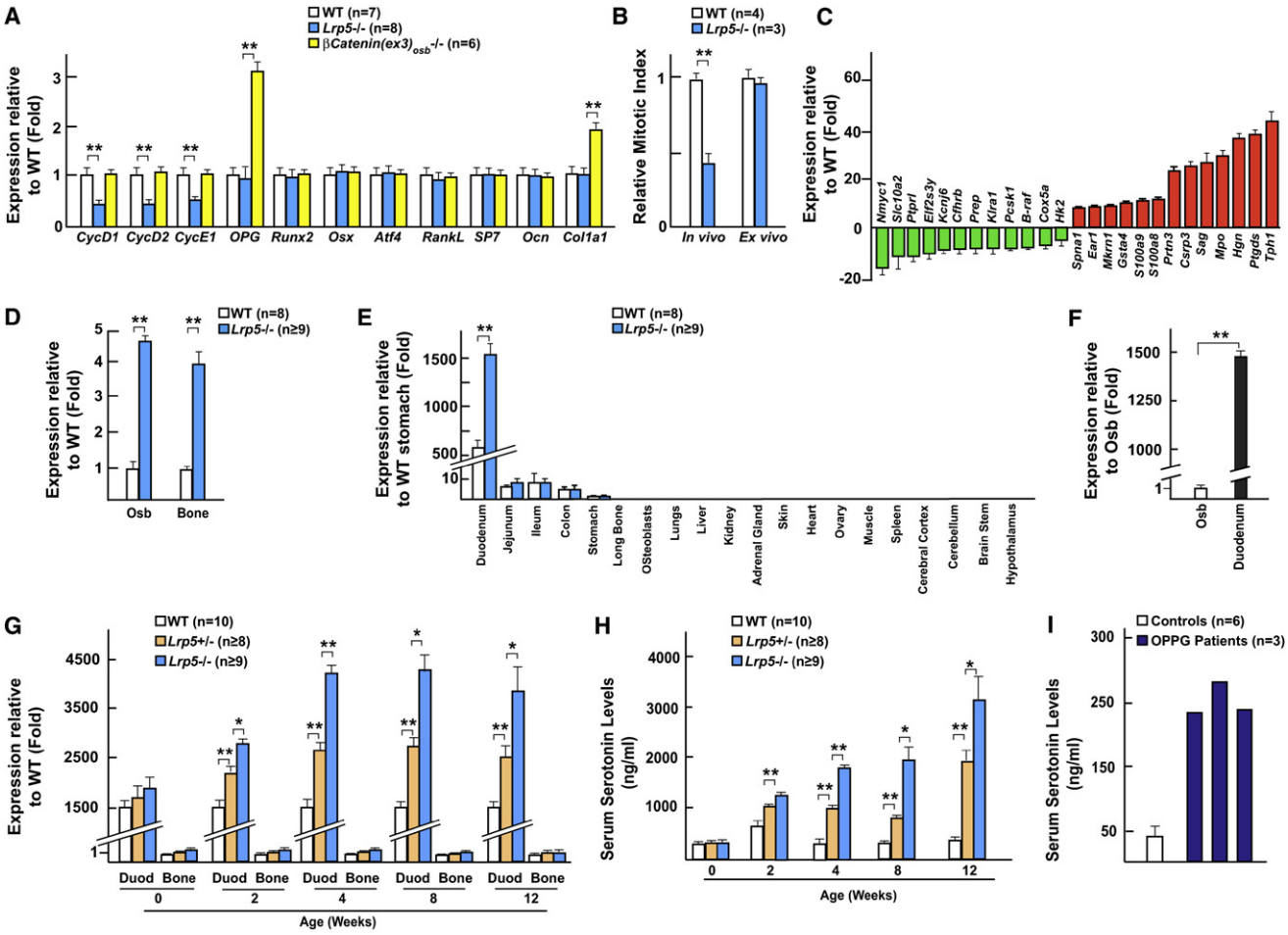



**Figure 1. Increased *Tph1* Expression and Serum Serotonin Levels in *Lrp5*$^{-/-}$ Mice**

(A) Real-time PCR analysis of gene expression in WT, *Lrp5*$^{-/-}$, and *β-Cat(ex3)*$_{osb}$$^{-/-}$ bones.

(B) Proliferation analysis (BrdU labeling) in vivo and ex vivo of WT and *Lrp5*$^{-/-}$ osteoblasts.

(C) Microarray analysis of WT and *Lrp5*$^{-/-}$ bones.

(D) Increased *Tph1* expression in osteoblasts (Osb) and bones of *Lrp5*$^{-/-}$ mice by real-time PCR analysis.

(E and F) Real-time PCR analysis of *Tph1* expression in tissues of WT and *Lrp5*$^{-/-}$ mice (E), and in primary osteoblasts (Osb) and gut of WT mice (F).

(G and H) Real-time PCR analysis of *Tph1* expression in gut versus bone (G), and serum serotonin levels (H) in WT, *Lrp5*$^{+/-}$, and *Lrp5*$^{-/-}$ mice at indicated ages.

(I) Serum serotonin levels in two OPPG patients and age-matched controls (n = 6).

Results are given as means ± standard deviations (SDs). Statistical analysis was performed by Student's t test. For all panels, *p < 0.05 and **p < 0.01 versus WT or control.

bone phenotype of the *Lrp5*$^{-/-}$ mice, we relied on cell-based and in vivo experiments.

To elucidate the discrepancy between the paucity of osteoblasts in *Lrp5*$^{-/-}$ bones but their normal proliferation ability ex vivo, we tested whether serotonin is an inhibitor of osteoblast proliferation. When treated with serotonin for 24 hr, proliferation of WT or *Lrp5*$^{-/-}$ osteoblasts was decreased to the same extent (Figure 2A); in contrast, Wnt3A-conditioned media did not affect osteoblast proliferation (Figure S2F). Serotonin decreased expression of *CycD1*, *D2*, and *E1* without affecting expression of *Type I Collagen* or of other genes characteristic of the osteoblast phenotype (Figures 2B and S2A), a gene expression profile remarkably similar to the one seen in the *Lrp5*$^{-/-}$ bones (Figure 1A). To further determine whether serotonin inhibits osteoblast proliferation in a Wnt-dependent or -independent manner, we treated ROS17 osteoblastic cells transfected with the TOPFLASH reporter construct with increasing amounts of serotonin or, as a positive control, with lithium chloride (LiCl) (Clement-Lacroix et al., 2005). Whereas LiCl increased the activity of this reporter vector in all experiments, serotonin always failed to do so, indicating that the serotonin and canonical Wnt signaling pathways are distinct in osteoblasts (Figure 2C).

If the increase in blood serotonin level is a cause of the *Lrp5*$^{-/-}$ mice bone phenotype, then normalizing it should also normalize bone mass in these mice. To test this hypothesis, we performed two in vivo experiments. Because serotonin is a tryptophan derivative, we first fed WT and *Lrp5*$^{-/-}$ mice from 3 to 12 weeks of age a diet containing 75% less tryptophan than a normal diet.

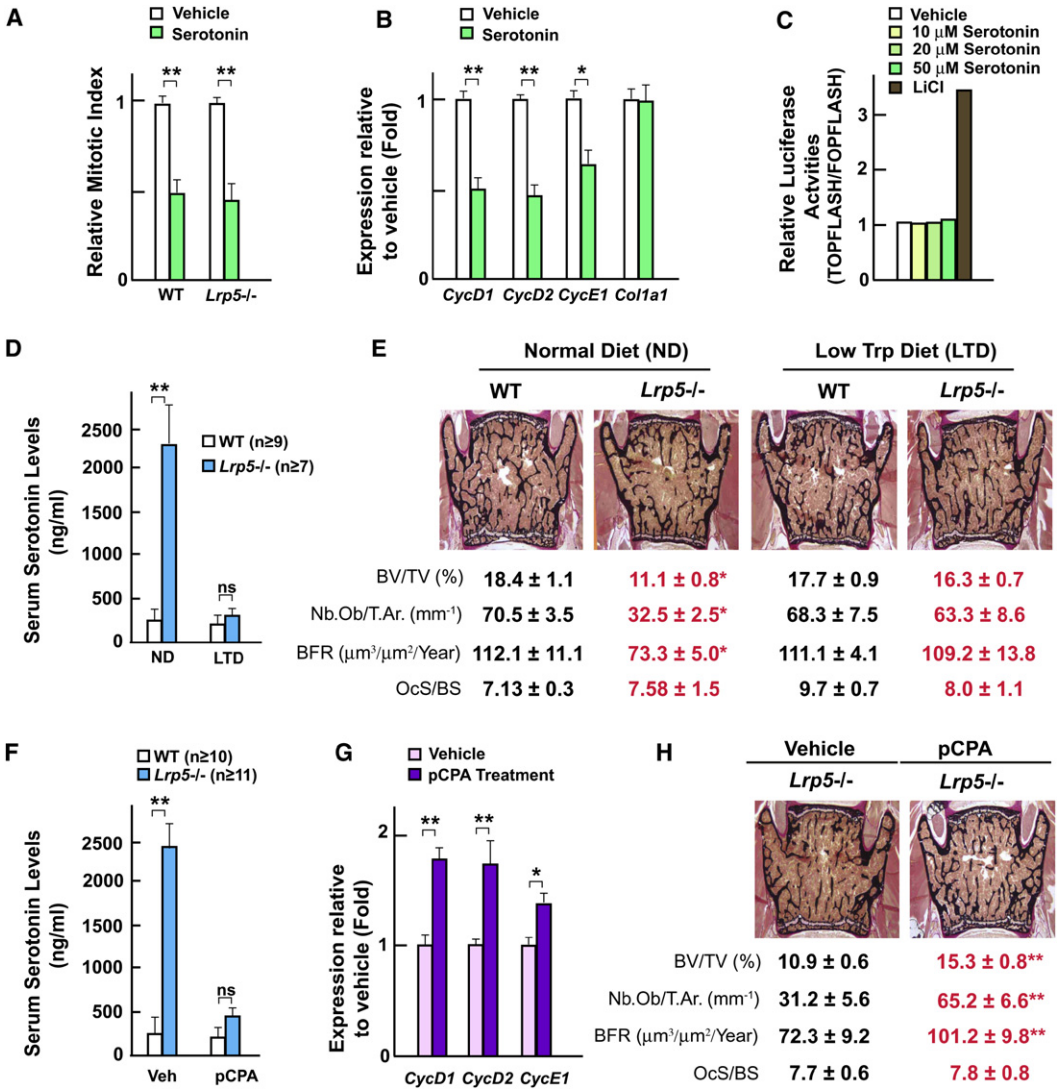



**Figure 2. Serotonin Inhibits Osteoblast Proliferation, and Decreasing Serum Serotonin Levels Correct *Lrp5*$^{-/-}$ Mice Bone Phenotype**

(A) Ex vivo proliferation rates of WT and *Lrp5*$^{-/-}$ osteoblasts treated with serotonin (50 μM) for 24 hr.

(B) Real-time PCR analysis of *Cyclins* and *Col1a1* expression in WT osteoblasts treated with serotonin (50 μM) for 24 hr.

(C) TOPFLASH reporter activities in osteoblasts in response to serotonin (10–50 μM) or lithium chloride (LiCl, 10 mM).

(D and E) Serum serotonin levels (D) and histological analysis of vertebrae (E) of WT and *Lrp5*$^{-/-}$ mice fed a normal diet or a 75% tryptophan-less diet. Mineralized bone matrix is stained in black by von Kossa reagent. Histomorphometric parameters: BV/TV, bone volume over trabecular volume; Nb.Ob/T.Ar., number of osteoblasts per trabecular area; BFR, bone formation rate; OcS/BS, osteoclast surface per bone surface.

(F–H) Serum serotonin levels (F), real-time PCR analysis of *Cyclin* expression in bone (G), and histomorphometric analyses (H) in WT and *Lrp5*$^{-/-}$ mice treated with the serotonin synthesis inhibitor pCPA (100 mg/kg).

Results are given as means ± SDs. Statistical analysis was performed by Student's t test. For all panels, *$p < 0.05$ and **$p < 0.01$ versus WT or control.

This reduction in tryptophan intake decreased circulating serotonin levels 8- to 10-fold without affecting serotonin brain content (Figures 2D and S2B) and normalized bone formation parameters and bone mass in *Lrp5*$^{-/-}$ mice (Figure 2E). Second, 4-week-old WT and *Lrp5*$^{-/-}$ mice were administered para-chlorophenylalanine (pCPA), a drug inhibiting serotonin synthesis, for 8 weeks (Kubera et al., 2000). pCPA decreased circulating serotonin levels 5- to 7-fold in *Lrp5*$^{-/-}$ mice without affecting their serotonin brain content; it also normalized in *Lrp5*$^{-/-}$ mice bone mass, bone formation parameters, and *Cyclin* expression in bones (Figures 2F–2H and S2C). That pCPA did not induce regression of the hyaloid vessels in the eyes suggests that the eye phenotype of the *Lrp5*$^{-/-}$ mice is not caused by this increase in circulating serotonin levels (Figures S2D and S2E).

Taken together, results of the cell biology experiments of the low tryptophan diet and the pCPA-induced serotonin depletion in WT and *Lrp5*$^{-/-}$ mice indicate that higher blood serotonin levels contribute to the decrease in bone formation and bone mass in *Lrp5*$^{-/-}$ mice.

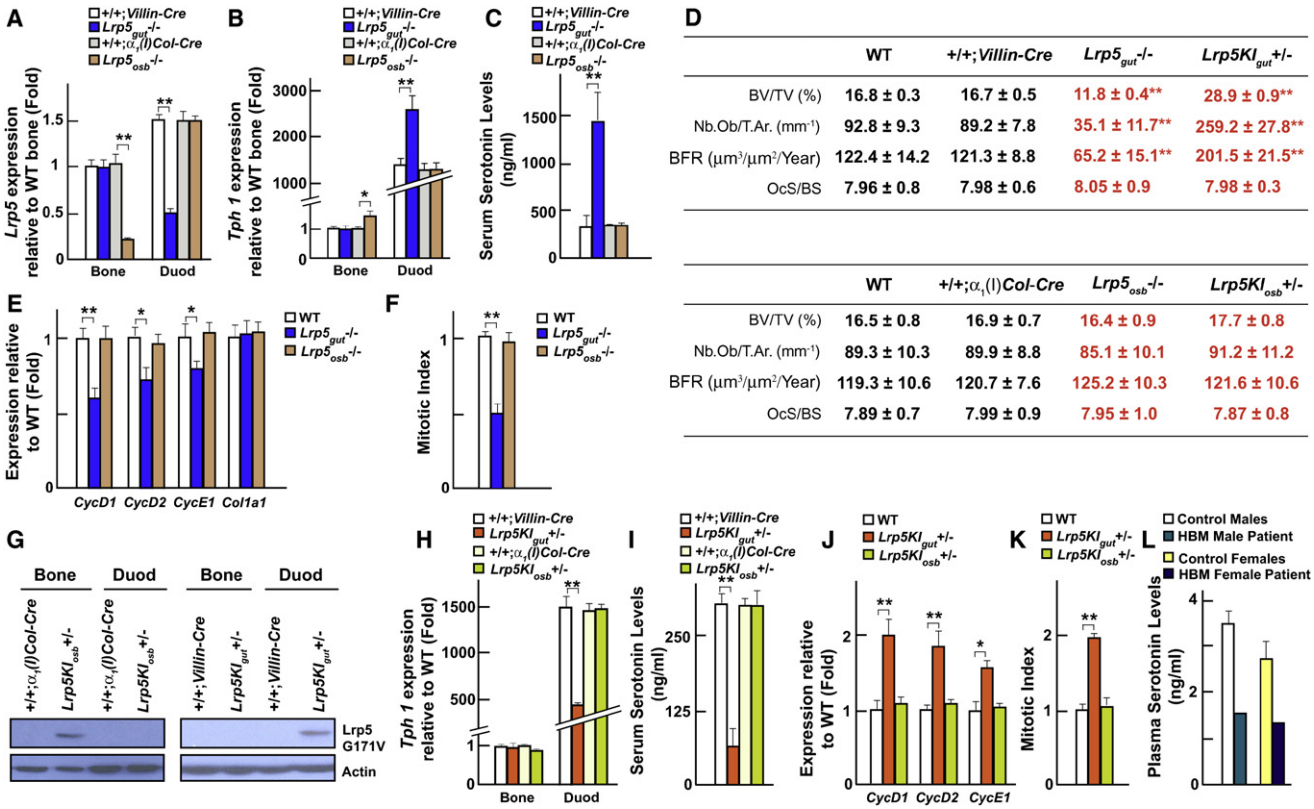



**Figure 3. Lrp5 Regulates Serotonin Synthesis through Its Expression in Gut**

(A and B) Real-time PCR analysis of *Lrp5* (A) and *Tph1* (B) expression in the gut and long bones (Bone) of $Lrp5_{gut}^{-/-}$ and $Lrp5_{osb}^{-/-}$ compared to +/+;*Villin-Cre* and +/+;$\alpha_1$*(I)Col-Cre* mice, respectively.

(C) Serum serotonin levels in +/+;*Villin-Cre*, $Lrp5_{gut}^{-/-}$, +/+;$\alpha_1$*(I)Col-Cre*, and $Lrp5_{osb}^{-/-}$ mice.

(D) Histomorphometric analysis of vertebrae of WT, +/+;*Villin-Cre*, $Lrp5_{gut}^{-/-}$, $Lrp5KI_{gut}^{+/-}$, +/+;$\alpha_1$*(I)Col-Cre*, $Lrp5_{osb}^{-/-}$, and $Lrp5KI_{osb}^{+/-}$ mice.

(E) Real-time PCR analysis of *Cyclins* and *Col1a1* expression in long bones of WT, $Lrp5_{gut}^{-/-}$, and $Lrp5_{osb}^{-/-}$ mice.

(F) In vivo osteoblast proliferation in WT, $Lrp5_{gut}^{-/-}$, and $Lrp5_{osb}^{-/-}$ mice.

(G) Western blot analysis of Lrp5 high bone mass (G171V) cDNA expression (Flag) in bone and gut of $Lrp5KI_{osb}^{+/-}$ and $Lrp5KI_{gut}^{+/-}$ compared to +/+;$\alpha_1$*(I)Col-Cre* and +/+;*Villin-Cre* mice, respectively.

(H and I) Real-time PCR analysis of *Tph1* expression (H) in the gut and long bones (Bone) and serum serotonin levels (I) in $Lrp5KI_{osb}^{+/-}$ and $Lrp5KI_{gut}^{+/-}$ compared to +/+;*Villin-Cre* and +/+;$\alpha_1$*(I)Col-Cre* mice, respectively.

(J and K) Real-time PCR analysis of *Cyclins* and *Col1a1* expression (J) and in vivo osteoblast proliferation (K) in long bones of WT, $Lrp5KI_{gut}^{+/-}$, and $Lrp5KI_{osb}^{+/-}$ mice.

(L) Plasma serotonin levels in two high bone mass (HBM) patients and age-matched controls (n = 3).

Results are given as means ± SDs. Statistical analysis was performed by Student's t test. For all panels, *p < 0.05 and **p < 0.01 versus WT or control.

## Lrp5 Regulates Serotonin Synthesis and Bone Formation through Its Duodenal Expression

In light of these results, we next asked whether *Lrp5* regulates *Tph1* expression, serotonin synthesis, and bone formation through its duodenal or its osteoblast expression. To that end, we generated mice harboring a floxed loss-of-function allele of *Lrp5* (Figures S3A and S3B) and crossed them with either *Villin-Cre* transgenic mice to delete *Lrp5* from gut cells ($Lrp5_{gut}^{-/-}$) or with $\alpha_1$*(I)Col-Cre* transgenic mice to delete it from osteoblasts only ($Lrp5_{osb}^{-/-}$) (Dacquin et al., 2002; el Marjou et al., 2004). Real-time PCR analysis demonstrated that efficient recombination occurred in duodenal cells but not in osteoblasts in the $Lrp5_{gut}^{-/-}$ mice, whereas the opposite was true in the $Lrp5_{osb}^{-/-}$ mice (Figures 3A and S3C). *Tph1* expression was high in the gut and normal in osteoblasts in $Lrp5_{gut}^{-/-}$ mice; the opposite was true in the $Lrp5_{osb}^{-/-}$ mice (Figure 3B). Circulating serotonin levels were 5- to 8-fold higher in $Lrp5_{gut}^{-/-}$ than in WT mice but remained normal in $Lrp5_{osb}^{-/-}$ mice (Figure 3C). In both mutant mice, brain serotonin content was normal (data not shown).

Histomorphometric analyses showed a decrease in bone mass in $Lrp5_{gut}^{-/-}$ but not in $Lrp5_{osb}^{-/-}$ mice (Figure 3D). This low bone mass was secondary to a decrease in osteoblast numbers and bone formation whereas osteoclast numbers were not affected (Figure 3D). Expression of *CycD1*, *D2*, and *E1* was decreased in the bones of $Lrp5_{gut}^{-/-}$ mice but was unaffected in $Lrp5_{osb}^{-/-}$ bones, and BrdU incorporation assays showed that osteoblast proliferation was decreased in $Lrp5_{gut}^{-/-}$ but not in $Lrp5_{osb}^{-/-}$ mice (Figures 3E and 3F). Thus, gut- but not osteoblast-specific deletion of *Lrp5* recapitulates the low bone

mass and the molecular abnormalities observed in the *Lrp5*$^{-/-}$ mice. As is the case for *Lrp5*$^{+/-}$ mice, *Lrp5*$_{gut}$$^{+/-}$ mice displayed a low bone formation/low bone mass phenotype (Figure S3D). These data suggest that Lrp5 regulates bone formation through its duodenal expression. In contrast, histological examination failed to detect any persistence of hyaloid vessels of the eyes in either *Lrp5*$_{gut}$$^{-/-}$ or *Lrp5*$_{osb}$$^{-/-}$ mice (Figure S3E). This latter result, along with the inability of pCPA to prevent appearance of the eye phenotype in the *Lrp5*$^{-/-}$ mice, demonstrates that Lrp5 uses different mechanisms to regulate bone formation and eye vascularization.

## Duodenal-Specific *Lrp5*-Activating Mutation Causes High Bone Mass

The demonstration that *Lrp5* loss of function affects bone mass by enhancing serotonin synthesis in the enterochromaffin cells raised the prospect that, conversely, *Lrp5* gain-of-function mutations might affect bone mass by decreasing serotonin synthesis in these cells. To test this hypothesis, we generated mice harboring a floxed allele of *Lrp5* that included the mutation causing high bone mass in humans (Boyden et al., 2002) (Figures S3F and S3G). These mice were crossed with either *Villin-Cre* or $\alpha_1$*(I)Col-Cre* transgenic mice to generate mice expressing this *Lrp5* gain-of-function mutation in gut cells (*Lrp5KI*$_{gut}$$^{+/-}$) or in osteoblasts (*Lrp5KI*$_{osb}$$^{+/-}$). We studied heterozygous mutant mice because high bone mass syndrome is observed in patients heterozygous for the mutation (Boyden et al., 2002).

*Lrp5KI*$_{gut}$$^{+/-}$ mice expressed the mutated protein in gut but not in osteoblasts; the opposite was true for *Lrp5KI*$_{osb}$$^{+/-}$ mice (Figure 3G, S3H, and S3I). *Tph1* expression in gut and circulating serotonin levels were low in *Lrp5KI*$_{gut}$$^{+/-}$ mice (Figures 3H and 3I) whereas serotonin content in the brain was not affected (data not shown). Histomorphometric analyses showed that bone formation parameters and bone mass were high in *Lrp5KI*$_{gut}$$^{+/-}$ but normal in *Lrp5KI*$_{osb}$$^{+/-}$ mice (Figure 3D). Expression of *Cyclins* and in vivo osteoblast proliferation were increased in *Lrp5KI*$_{gut}$$^{+/-}$ bones; none of these parameters were modified in *Lrp5KI*$_{osb}$$^{+/-}$ mice (Figures 3J, 3K, and S3J). To validate our findings obtained in mice, we studied two patients with high bone mass caused by the same activating mutation (G171V) in *LRP5* (Boyden et al., 2002) and observed a 50% decrease in circulating serotonin levels (Figure 3L).

In summary, based on the analyses of cell-specific loss- and gain-of-function mutations of *Lrp5* models, it appears that this gene regulates bone formation through its duodenal but not its bone expression.

## Gut-Derived Serotonin Regulates Bone Formation

To further demonstrate that it is duodenal-derived serotonin that regulates bone formation, we generated mice harboring a floxed loss-of-function allele of *Tph1* and crossed them with *Villin-Cre* or $\alpha_1$*(I)Col-Cre* mice to generate *Tph1*$_{gut}$$^{-/-}$ or *Tph1*$_{osb}$$^{-/-}$ mice (Figures S4A–S4C). Because *Tph1* is overexpressed in *Lrp5*$^{-/-}$ mice, its deletion in the appropriate cell type should result in a bone phenotype opposite the one of the *Lrp5*$^{-/-}$ mice (i.e., high bone mass). *Tph1*$_{gut}$$^{-/-}$ mice had a 10-fold reduction in *Tph1* expression in gut, whereas *Tph1* expression in bone and osteoblasts was not affected; conversely, *Tph1*$_{osb}$$^{-/-}$ mice had a 10-fold reduction in *Tph1* expression in bones and osteoblasts but a normal *Tph1* expression in gut (Figures 4A and 4B). Circulating serotonin levels were low in *Tph1*$_{gut}$$^{-/-}$ but normal in *Tph1*$_{osb}$$^{-/-}$ mice (Figure 4C).

As anticipated, mice lacking *Tph1* in the gut developed a severe high bone mass phenotype secondary to an increase in osteoblast number and bone formation rate; bone resorption parameters were not affected (Figure 4D). This phenotype, that is the mirror image of the one seen in the *Lrp5*$^{-/-}$ mice, was present in 4-, 6-, and 12-week-old *Tph1*$_{gut}$$^{-/-}$ mice (Figures 4D and S4D). In contrast, mice lacking *Tph1* in osteoblasts had only a marginal, nonsignificant increase in bone mass (Figure 4E). Expression of *CycD1*, *D2*, and *E1*, which was decreased in the absence of *Lrp5*, was increased in *Tph1*$_{gut}$$^{-/-}$ but normal in *Tph1*$_{osb}$$^{-/-}$ bones, and BrdU incorporation assays showed that osteoblast proliferation was increased in *Tph1*$_{gut}$$^{-/-}$ but not in *Tph1*$_{osb}$$^{-/-}$ mice (Figures 4F and 4G).

## The Htr1b Receptor Mediates Serotonin Regulation of Osteoblast Proliferation

If circulating serotonin is a hormone inhibiting bone formation, it must act through a specific receptor(s) present on osteoblasts whose inactivation should lead to an increase in bone formation and high bone mass.

Among the 14 known serotonin receptors, only 3 are expressed in osteoblasts: *Htr1b*, the most highly expressed, *Htr2b*, and *Htr2a* (Figure 5A). *Htr1b* is preferentially expressed in bone and osteoblasts outside the brain, whereas *Htr2b* and *Htr2a* have a broader pattern of expression (Figures 5B and S5A). Analysis of *Htr2a*$^{-/-}$ mice failed to detect any abnormalities in osteoblast number, bone formation, bone resorption, and bone mass at 1 or 3 months of age (Figures 5C and S5B), two time points at which *Lrp5*$^{-/-}$ mice already display a severe bone phenotype. The same was true for mice lacking in osteoblasts only, *Htr2b* (Figures 5D and S5B–S5E). In contrast, mice lacking either one or the two *Htr1b* alleles displayed a similar increase in osteoblast number, bone formation rate, and bone mass at those ages (Figures 5E and S5B). That haploinsufficiency at *Htr1b* has consequences as severe as the homozygous deletion, at least at 6 weeks of age, is consistent with the fact that heterozygous loss-of-function mutation in *Tph1* or heterozygous gain-of-function mutation in *Lrp5* also have consequences as severe on bone mass as homozygous mutations. Furthermore, it is also in accord with the observation that patients with high bone mass are only heterozygous for *Lrp5* mutations. Altogether, it demonstrates that loss of serotonin signaling exerts a dominant function on bone remodeling. Molecularly, expression of *CycD1*, *D2*, and *E1* that is downregulated in *Lrp5*$^{-/-}$ bones was upregulated in *Htr1b*$^{-/-}$ but not in *Htr2a*$^{-/-}$ or *Htr2b*$_{osb}$$^{-/-}$ bones, and serotonin treatment decreased *CycD1* expression in WT, *Htr2a*$^{-/-}$, and *Htr2b*$_{osb}$$^{-/-}$ but not in *Htr1b*$^{-/-}$ osteoblasts (Figures 5F and 5G). BrdU incorporation assays confirmed that osteoblast proliferation was increased in *Htr1b*$^{-/-}$ but not in *Htr2a*$^{-/-}$ and *Htr2b*$_{osb}$$^{-/-}$ bones (Figure 5H). Other osteoblast-specific genes such as *Osteoprotegerin*, a Wnt target gene (Glass et al., 2005), were normally expressed in *Htr1b*$^{-/-}$ bones, further differentiating canonical Wnt and serotonin signaling pathways in osteoblasts (Figure S5F).

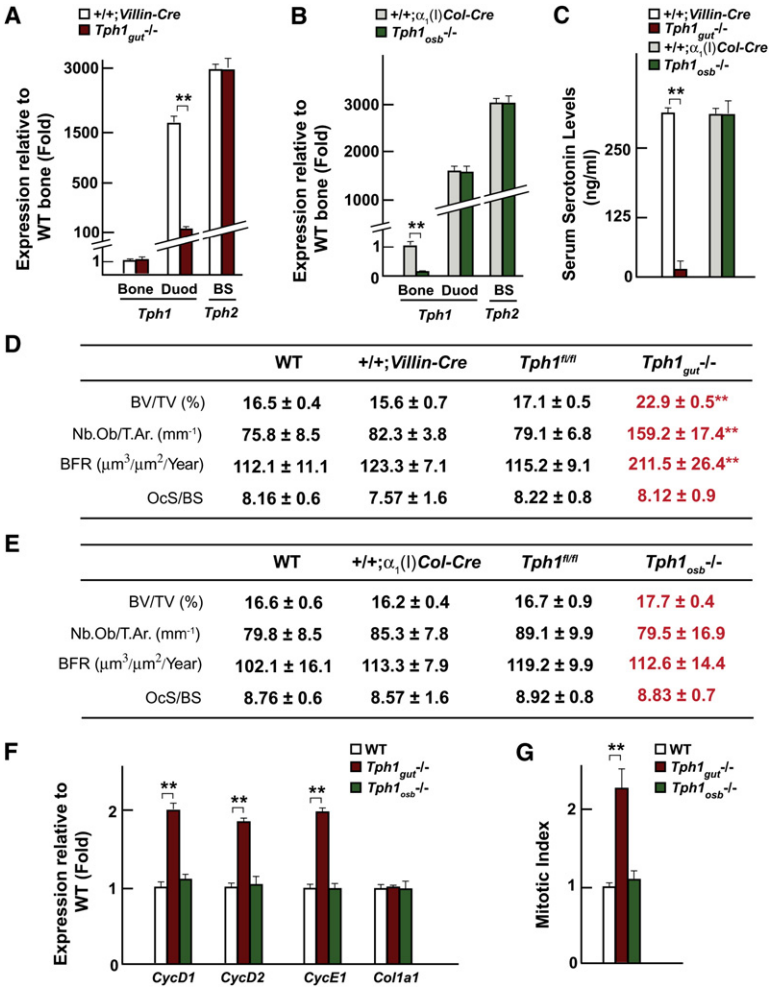



**Figure 4. Duodenal-Derived Serotonin Regulates Bone Formation**

(A and B) Real-time PCR analysis of *Tph1* expression in the gut and long bones (Bone) and *Tph2* expression in the brainstem (BS) of $Tph1_{gut}^{-/-}$ and $Tph1_{osb}^{-/-}$ compared to +/+;*Villin-Cre* and +/+;$\alpha_1$*(I)Col-Cre* mice, respectively.

(C–E) Serum serotonin levels (C) and bone histomorphometric analysis (vertebrae) (D and E) in WT, +/+;*Villin-Cre*, $Tph1_{gut}^{-/-}$, +/+;$\alpha_1$*(I)Col-Cre*, and $Tph1_{osb}^{-/-}$ mice.

(F) Real-time PCR analysis of *Cyclins* and *Col1a1* expression in long bones of WT, $Tph1_{gut}^{-/-}$, and $Tph1_{osb}^{-/-}$ mice.

(G) In vivo osteoblast proliferation in WT, $Tph1_{gut}^{-/-}$, and $Tph1_{osb}^{-/-}$ mice.

Results are given as means ± SDs. Statistical analysis was performed by Student's t test. For all panels, *$p < 0.05$ and **$p < 0.01$ versus WT or control.

To ascertain that *Htr1b* regulates bone formation through its expression in osteoblasts, we generated mice lacking *Htr1b* only in osteoblasts ($Htr1b_{osb}$-deficient mice; Figures S5G and S5H). As shown in Figure 5I, $Htr1b_{osb}^{+/-}$ mice display the same high bone formation/high bone mass phenotype as the one observed in the $Htr1b^{+/-}$ mice. Taken together, these results indicate that serotonin uses one predominant receptor, Htr1b, to affect osteoblast biology.

## Serotonin Regulates Osteoblast Proliferation through CREB

We next searched for the transcriptional mediator(s) of serotonin signaling in osteoblasts. To address this question, because loss-of-function mutations in most of the key transcription factors governing osteoblast differentiation are embryonic lethal, we generated compound mutant mice lacking one allele of *Lrp5* and one allele of each transcription factor of interest. We reasoned that if *Lrp5* and a particular transcription factor are in the same genetic cascade, compound heterozygous mice may reproduce the bone phenotype of the $Lrp5^{-/-}$ mice. Removing one allele of *Runx2* or *Osx* from $Lrp5^{+/-}$ mice did not worsen the bone phenotype of these latter mutant mice, a result consistent with the normal expression of *Runx2* and *Osx* in $Lrp5^{-/-}$ osteoblasts (Figure 1A). These observations strongly suggest that neither of these two factors act downstream of serotonin (Figures 6A and S6).

Because Htr1b is linked to the G$\alpha$*i* protein that inhibits cAMP production and PKA-dependent phosphorylation (Heath and Hen, 1995), we studied transcription factors that, like CREB, are phosphorylated by PKA. Removing one allele of *Atf4*, a CREB-related osteoblast-enriched transcription factor (Yang et al., 2004), from $Lrp5^{+/-}$ mice did not affect the bone phenotype of these latter mice, arguing against a role for ATF4 in mediating serotonin signaling in osteoblasts (Figures 6A and S6). In contrast, compound mutant mice lacking one allele of *Lrp5* and, in osteoblasts only, one allele of *Creb* displayed a low bone formation/low bone mass phenotype indistinguishable from the one observed in the $Lrp5^{-/-}$ mice (Figures 6A and S6). This result suggested that CREB is a major transcriptional mediator of serotonin signaling in osteoblasts. In support of this contention, serotonin treatment of primary osteoblasts decreased phosphorylation of CREB on Ser133, the target of PKA, in WT but not in $Htr1b^{-/-}$ cells (Figure 6B) and abolished binding of CREB to the promoter of *CycD1*, a known target gene of CREB (Figure 6C) (Fu et al., 2005). That *CycD1* expression is decreased in $Creb_{osb}^{+/-}$ but not in $Atf4^{+/-}$ mice, used here as a negative control, is in full agreement with the ex vivo assays presented above (Figure 6D). Furthermore, that *Creb* expression was decreased in $Lrp5_{gut}^{-/-}$ long bones, increased in $Lrp5KI_{gut}^{+/-}$ long bones, but unaffected in either $Lrp5_{osb}^{-/-}$ or $Lrp5KI_{osb}^{+/-}$ long bones (Figure 6E) raises the prospect that serotonin regulates *Creb* expression in vivo.

## Genetic Validation of the Interactions between *Lrp5*, *Tph1*, *Htr1b*, and *Creb*

To demonstrate that the gut-derived serotonin/osteoblast/CREB pathway described here mediates Lrp5-dependent regulation of osteoblast proliferation and bone formation, we generated various compound mutant mice. First, we showed that mutant

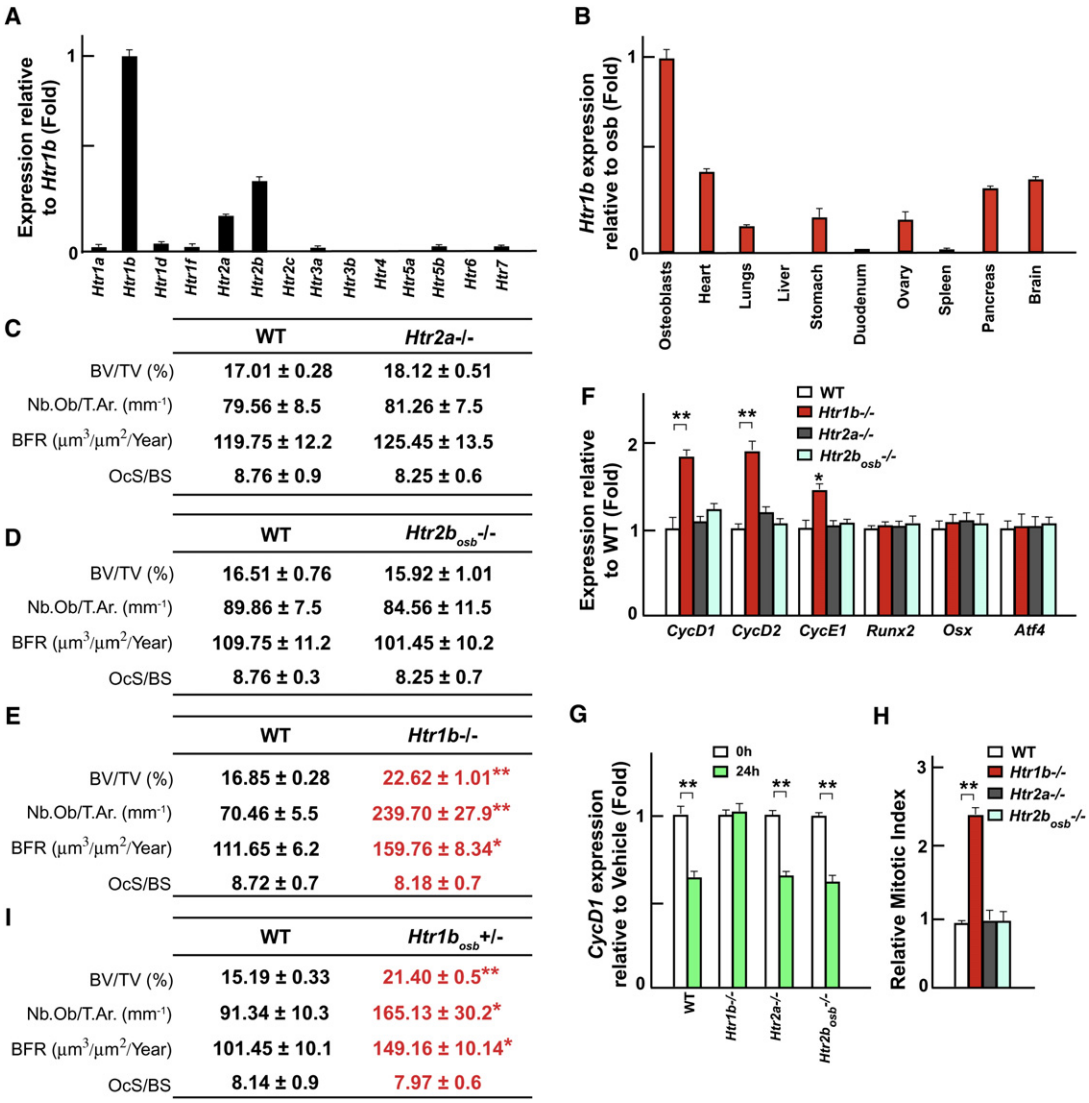



**Figure 5. Serotonin Inhibits Osteoblast Proliferation through Htr1b**

(A and B) Real-time PCR analysis of serotonin receptor expression in primary osteoblasts (A) and of *Htr1b* expression in different tissues of WT mice (B).
(C–E) Histomorphometric analysis of vertebrae of 3-month-old WT, *Htr2a*$^{-/-}$ (C), *Htr2b*$_{osb}$$^{-/-}$ (D), and *Htr1b*$^{-/-}$ (E) mice.
(F) Real-time PCR analysis of *Cyclins*, *Runx2*, *Osx*, and *Atf4* expression in long bones of WT, *Htr1b*$^{-/-}$, *Htr2a*$^{-/-}$, and *Htr2b*$_{osb}$$^{-/-}$ mice.
(G) Real-time PCR analysis of *CycD1* expression in primary osteoblasts of WT, *Htr1b*$^{-/-}$, *Htr2a*$^{-/-}$, and *Htr2b*$_{osb}$$^{-/-}$ mice treated with serotonin (50 µM).
(H) In vivo osteoblast proliferation in WT, *Htr1b*$^{-/-}$, *Htr2a*$^{-/-}$, and *Htr2b*$_{osb}$$^{-/-}$ mice.
(I) Histomorphometric analysis of vertebrae of 6-week-old WT and *Htr1b*$_{osb}$$^{+/-}$ mice.
Results are given as means ± SDs. Statistical analysis was performed by Student's t test. For all panels, *$p < 0.05$ and **$p < 0.01$ versus WT or control.

mice lacking one allele of *Htr1b* and one allele of *Tph1* in gut cells developed the same high bone mass phenotype as *Htr1b*$^{-/-}$ or *Tph1*$_{gut}$$^{-/-}$ mice (Figures 7A and S7A). Moreover, removal of one allele of *Creb*, in an osteoblast-specific manner, from the *Htr1b*$^{-/-}$ mice rescued the increase in bone formation parameters and the high bone mass of these mutant mice (Figures 7A and S7A). These experiments indicated that *Tph1* in gut cells, and *Htr1b* and *Creb* in osteoblasts, are in the same genetic cascade regulating bone formation. Next, to ascertain that *Tph1* in the gut and *Htr1b* mediate Lrp5-dependent regulation of bone mass, we removed from the *Lrp5*$^{-/-}$ mice one allele of *Tph1* in gut cells or one allele of *Htr1b* (Figures 7A and S7A). Both these manipulations could normalize the bone abnormalities observed in the *Lrp5*$^{-/-}$ mice, indicating that serotonin synthesis in duodenal enterochromaffin cells and serotonin binding to Htr1b are downstream of Lrp5 (Figures 7A and S7A). Thus, the results presented in this study indicate that the main mechanism whereby Lrp5 favors bone formation and bone mass accrual is by inhibiting *Tph1* expression in and serotonin synthesis by enterochromaffin cells of the gut. Serotonin in turn prevents bone formation following its binding to Htr1b by inhibiting *Creb* expression and function, *CycD1* expression, and osteoblast proliferation.

A

| | WT | *Lrp5* +/- | *Runx2* +/- | *Lrp5* +/-; *Runx2* +/- | *Osx* +/- | *Lrp5* +/-; *Osx* +/- | *Atf4* +/- | *Lrp5* +/-; *Atf4* +/- | $Creb_{osb}$ +/- | *Lrp5* +/-; $Creb_{osb}$+/- |
|---|---|---|---|---|---|---|---|---|---|---|
| BV/TV (%) | 16.1 ± 0.45 | 13.5 ± 0.4* | 15.8 ± 0.35 | 12.9 ± 0.32 | 16.1 ± 0.28 | 13.5 ± 0.4 | 15.2 ± 0.77 | 12.9 ± 0.95 | 15.5 ± 1.22 | 9.8 ± 0.36* |
| Nb.Ob/T.Ar. ($mm^{-1}$) | 76.6 ± 9.2 | 56.8 ± 7.1* | 66.2 ± 6.1 | 54.7 ± 3.1 | 66.2 ± 21.1 | 56.7 ± 7.1 | 57.6 ± 6.1 | 53.7 ± 10.1 | 63.6 ± 4.2 | 32.7 ± 7.4* |
| BFR ($\mu m^3/\mu m^2$/Year) | 104.3 ± 12.1 | 67.0 ± 8.9** | 97.3 ± 10.9 | 65.0 ± 7.9 | 105.3 ± 10.9 | 75.0 ± 7.9 | 59.2 ± 7.9 | 75.5 ± 16.5 | 62.7 ± 7.3 | 40.4 ± 6.1** |
| OcS/BS | 7.76 ± 0.4 | 7.92 ± 0.3 | 7.75 ± 0.4 | 7.68 ± 0.9 | 7.59 ± 0.8 | 7.59 ± 0.5 | 7.85 ± 0.9 | 7.57 ± 0.4 | 7.83 ± 0.5 | 7.52 ± 0.4 |

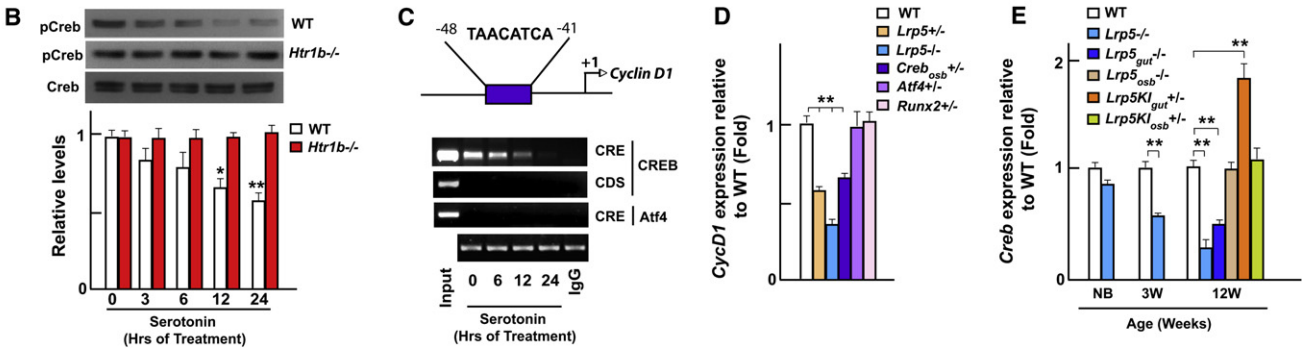



**Figure 6. Lrp5 Mediates Its Effect through CREB**

(A) Histomorphometric analysis of vertebrae of WT and single ($Lrp5^{+/-}$, $Runx2^{+/-}$, $Osx^{+/-}$, $Atf4^{+/-}$, or $Creb_{osb}{}^{+/-}$) and double ($Lrp5^{+/-}$;$Runx2^{+/-}$, $Lrp5^{+/-}$;$Osx^{+/-}$, $Lrp5^{+/-}$;$Atf4^{+/-}$, or $Lrp5^{+/-}$;$Creb_{osb}{}^{+/-}$) heterozygous mutant mice.

(B and C) Western blot analysis of CREB phosphorylation (B) and chromatin immunoprecipitation assay of CREB binding to the consensus cAMP response element (CRE) in the *CycD1* promoter (C) at 0, 3, 6, 12, and 24 hr upon serotonin (50 μM) treatment. Coding sequence (CDS) PCR, IgG pull-down, and ATF4 binding to the same site were used as negative controls.

(D) Real-time PCR analysis of *CycD1* expression in the long bones of WT, $Lrp5^{+/-}$, $Lrp5^{-/-}$, $Creb_{osb}{}^{+/-}$, $Osx^{+/-}$, and $Runx2^{+/-}$ mice.

(E) Real-time PCR analysis of *Creb* expression in the long bones of WT, $Lrp5^{-/-}$, $Lrp5_{gut}{}^{-/-}$, $Lrp5_{osb}{}^{-/-}$, $Lrp5KI_{gut}{}^{+/-}$, and $Lrp5KI_{osb}{}^{+/-}$ mice.

Results are given as means ± SDs. Statistical analysis was performed by Student's t test. For all panels, *$p < 0.05$ and **$p < 0.01$ versus WT or control.

### *Lrp5* and *β-Cat* Expression in Gut and Bone Mass

Because Lrp5 has been proposed to be a Wnt coreceptor, we next asked whether it was regulating *Tph1* expression in a β-Cat-dependent or -independent manner. To that end, we generated mice lacking one allele of *Lrp5* and one allele of *β-Cat* in gut cells (Figures S7B and S7C), reasoning that if Lrp5 and β-Cat were in the same genetic pathway then these compound heterozygous mice should have the same low bone formation and low bone mass phenotype as the $Lrp5^{-/-}$ mice. This strategy has been used by us to demonstrate genetic interaction between *β-Cat* and other genes in osteoblasts (Glass et al., 2005).

As shown in Figure 7B, whereas $Lrp5^{+/-}$ mice had a low bone formation and low bone mass phenotype, $\beta\text{-}Cat_{gut}{}^{+/-}$ mice did not; more importantly, the compound heterozygous $Lrp5^{+/-}$;$\beta\text{-}Cat_{gut}{}^{+/-}$ did not have a more severe low bone formation and low bone mass than the $Lrp5^{+/-}$ mice. Molecularly, there was no change in *Tph1* expression in the gut and in serum serotonin levels in $\beta\text{-}Cat_{gut}{}^{+/-}$ compared to WT mice (Figure 7C). We also generated compound mutant mice lacking one allele of *Tph1* and one allele of *β-Cat* in gut. Here again, we did not see in $Tph1_{gut}{}^{+/-}$;$\beta\text{-}Cat_{gut}{}^{+/-}$ any worsening of the bone phenotype observed in the $Tph1_{gut}{}^{+/-}$ mice (Figure 7B). Because of their negative nature, these results have to be interpreted cautiously, yet that *β-Cat* haploinsufficiency can in other cell types lead to phenotypic abnormalities strongly suggests that Lrp5 regulates *Tph1* expression and serotonin synthesis independently of *β-Cat* expression in gut (Glass et al., 2005).

### Decreasing Duodenal Serotonin Synthesis Protects Mice from Ovariectomy-Induced Bone Loss

To assess the biological importance of the serotonin regulation of bone mass, we tested whether the increase in bone formation parameters observed in $Tph1_{gut}{}^{-/-}$ mice was sufficient to protect them from ovariectomy-induced bone loss. WT and $Tph1_{gut}{}^{-/-}$ were ovariectomized (Ovx) at 6 weeks of age and analyzed at 3 months of age. Histomorphometric analyses showed an increase in bone resorption parameters in both WT and $Tph1_{gut}{}^{-/-}$ mice following Ovx (Figure 7D). Nevertheless, whereas Ovx WT mice developed a low bone mass phenotype, Ovx $Tph1_{gut}{}^{-/-}$ mice still harbored a high bone mass phenotype which was due to their increase in bone formation parameters (Figure 7D). Thus, decreasing serotonin production in enterochromaffin cells of the gut protects mice from ovariectomy-induced osteoporosis.

## DISCUSSION

This study uncovers an unanticipated molecular mechanism accounting for the Lrp5 regulation of bone formation; our findings

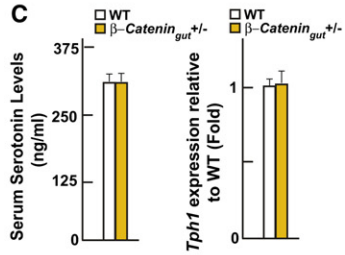
A

| | WT | *Htr1b+/-* | *Tph1$_{gut}$+/-* | *Tph1$_{gut}$+/-;Htr1b+/-* | *Htr1b -/-* | *Htr1b -/-;Creb$_{osb}$ +/-* | *Lrp5-/-* | *Lrp5-/-;Htr1b+/-* | *Lrp5-/-;Tph1$_{gut}$+/-* |
|---|---|---|---|---|---|---|---|---|---|
| BV/TV (%) | 17.0 ± 0.3 | 19.2 ± 0.6 | 21.0 ± 1.0* | 25.3 ± 0.6** | 24.3 ± 0.9 | 17.7 ± 0.7 | 11.6 ± 0.4 | 16.8 ± 0.4** | 15.9 ± 0.7* |
| Nb.Ob/T.Ar. (mm$^{-1}$) | 89.6 ± 5.3 | 112.6 ± 5.3 | 134.6 ± 9.3** | 234.6 ± 11.2** | 274.4 ± 27.1 | 99.2 ± 9.9 | 31.5 ± 6.5 | 86.9 ± 10.1** | 87.3 ± 11.9** |
| BFR (μm$^3$/μm$^2$/Year) | 119.7 ± 6.2 | 139.7 ± 6.2 | 149.7 ± 9.2* | 223.2 ± 8.1** | 223.2 ± 8.1 | 121.6 ± 9.8 | 74.7 ± 9.8 | 111.7 ± 16.1** | 109.7 ± 7.2** |
| OcS/BS | 7.93 ± 0.5 | 7.63 ± 0.6 | 7.53 ± 0.5 | 7.62 ± 0.5 | 7.81 ± 0.9 | 7.65 ± 0.4 | 7.61 ± 0.4 | 7.81 ± 0.8 | 7.63 ± 0.6 |

B

| | WT | β-*Catenin$_{gut}$+/-* | *Tph1$_{gut}$+/-* | *Tph1$_{gut}$+/-;*β-*Catenin$_{gut}$+/-* | *Lrp5+/-* | *Lrp5+/-;*β-*Catenin$_{gut}$+/-* |
|---|---|---|---|---|---|---|
| BV/TV (%) | 17.7 ± 0.4 | 18.1 ± 0.9 | 20.9 ± 0.9* | 20.1 ± 1.1 | 12.3 ± 0.8* | 13.6 ± 0.5 |
| Nb.Ob/T.Ar. (mm$^{-1}$) | 88.9 ± 9.4 | 99.6 ± 10.3 | 133.7 ± 8.6* | 129.1 ± 9.2 | 61.5 ± 8.7* | 67.6 ± 9.4 |
| BFR (μm$^3$/μm$^2$/Year) | 114.4 ± 9.9 | 116.5 ± 6.2 | 159.5 ± 10.1** | 149.3 ± 14.2 | 65.7 ± 9.2** | 64.2 ± 7.1 |
| OcS/BS | 7.53 ± 0.8 | 7.79 ± 0.8 | 7.64 ± 0.9 | 7.74 ± 0.5 | 7.84 ± 0.6 | 7.62 ± 0.5 |

C




D

| | WT | WT(Ovx) | *Tph1$_{gut}$-/-* | *Tph1$_{gut}$-/-*(Ovx) |
|---|---|---|---|---|
| BV/TV (%) | 16.2 ± 0.8 | 11.1 ± 0.6** | 22.6 ± 0.9 | 19.5 ± 1.1** |
| Nb.Ob/T.Ar. (mm$^{-1}$) | 99.3 ± 9.6 | 127.8 ± 9.3* | 167.6 ± 10.3 | 175.6 ± 9.2 |
| BFR (μm$^3$/μm$^2$/Year) | 103.5 ± 6.2 | 129.5 ± 13.2* | 228.9 ± 12.1 | 239.5 ± 7.5* |
| OcS/BS | 7.65 ± 0.9 | 13.59 ± 0.5** | 7.93 ± 0.6 | 13.32 ± 1.9** |

E

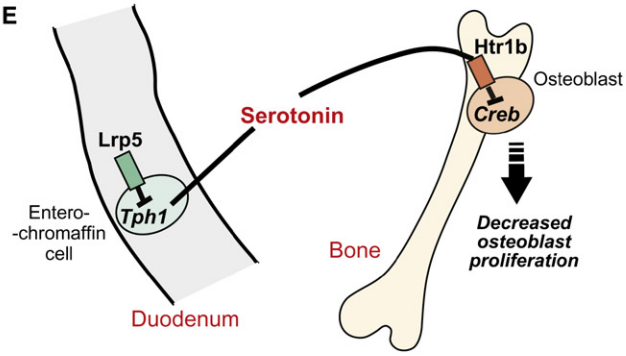



**Figure 7. Genetic Interaction between Lrp5 and Serotonin**

(A) Bone histomorphometric analysis (vertebrae) of WT, *Htr1b*$^{+/-}$, *Tph1$_{gut}$*$^{+/-}$, *Htr1b*$^{+/-}$;*Tph1$_{gut}$*$^{+/-}$, *Lrp5*$^{-/-}$, *Lrp5*$^{-/-}$;*Htr1b*$^{+/-}$, and *Lrp5*$^{-/-}$;*Tph1$_{gut}$*$^{+/-}$ mice.

(B) Bone histomorphometric analysis (vertebrae) of WT, *β-Cat$_{gut}$*$^{+/-}$, *Tph1$_{gut}$*$^{+/-}$;*β-Cat$_{gut}$*$^{+/-}$, *Lrp5*$^{+/-}$, and *Lrp5*$^{+/-}$;*β-Cat$_{gut}$*$^{+/-}$ mice.

(C) Serum serotonin levels and real-time PCR analysis of *Tph1* expression in the gut of WT and *β-Cat$_{gut}$*$^{+/-}$ mice.

(D) Bone histomorphometric analysis (vertebrae) of WT, WT(Ovx), *Tph1$_{gut}$*$^{-/-}$, and *Tph1$_{gut}$*$^{-/-}$ (Ovx) mice.

(E) Model of the Lrp5-dependent regulation of bone formation. Lrp5 favors bone formation and bone mass accrual by inhibiting *Tph1* expression and serotonin synthesis in enterochromaffin cells. Following its binding to Htr1b, serotonin inhibits *Creb* expression and function, which results in a decrease in *CycD1* expression and osteoblast proliferation.

Results are given as means ± SDs. Statistical analysis was performed by Student's t test. For all panels, *$p < 0.05$ and **$p < 0.01$ versus WT or control.

shift the emphasis from a paracrine to an endocrine regulation of bone mass, and from bone cells to enterochromaffin cells of the gut (Figure 7E). Moreover, given the cell-specific expression of *Tph1*, this study points toward novel therapeutic avenues for diseases characterized by a relative or absolute decrease in bone formation.

## Lrp5-Dependent Serotonin Synthesis in the Gut and Regulation of Bone Formation

Regardless of the mode of action of Lrp5, a hallmark of *Lrp5*$^{-/-}$ bones is a paucity of osteoblasts, a feature contrasting strikingly with the fact that *Lrp5*$^{-/-}$ osteoblasts proliferate normally ex vivo. One possible explanation of this discrepancy could be that osteoblast proliferation in the *Lrp5*$^{-/-}$ mice is affected by an extracellular signal that is not originating from osteoblasts themselves.

In full support of the hypothesis that the extracellular signal regulating osteoblast proliferation in the absence of *Lrp5* does not emanate from osteoblasts, cell-specific gene inactivation or gain-of-function studies established that it is through its expression in enterochromaffin cells of the gut and not through its expression in osteoblasts that *Lrp5* controls *Tph1* expression, serotonin synthesis, osteoblast proliferation, and bone formation. The differences in the severity of the bone phenotype observed in *Tph1$_{gut}$*$^{-/-}$ and *Lrp5KI$_{gut}$*$^{+/-}$ mice raises the hypothesis that Lrp5 regulates expression in enterochromaffin cells of other gene(s) regulating bone mass. Last, we cannot rule out that Lrp5 may exert an autocrine function in osteoblasts that was not detectable in the genetic manipulations performed here. By showing that OPPG and high bone mass syndrome are more gut- than bone-originating diseases, these results put new light on these two conditions. This work does provide an explanation for the osteoporosis often observed in patients

with autism who have high blood serotonin levels (Hediger et al., 2008); however, it does not explain why patients taking SSRIs chronically often develop osteoporosis (Richards et al., 2007).

### LRPs, Bone Remodeling, and Canonical Wnt Signaling

Lrp5 is a member of the family of lipoprotein receptor related proteins (Lrps), a small group of single-pass transmembrane proteins (Tamai et al., 2000). Lrp5 has a significant degree of sequence homology with the *Drosophila* protein Arrow, a Wingless coreceptor (Pinson et al., 2000; Tamai et al., 2004). Based on this sequence homology and multiple forced expression experiments in cell culture and in vivo, it has been assumed that Lrp5 is a coreceptor for Wnt proteins, the mammalian homologs of Wingless, and that it is in this capacity that it regulates bone formation (Gong et al., 2001; Kato et al., 2002).

However, at the present time, all in vivo evidence available indicates that Lrp5 regulates gut-derived serotonin production and bone formation in a canonical Wnt signaling pathway-independent manner. First, expression of classical Wnt target genes is normal in $Lrp5^{-/-}$ mice gut (Figure S7D); second, serotonin does not affect the activity of a TOPFLASH reporter construct or the expression of *Osteoprotegerin* in osteoblasts, nor does it affect osteoclast differentiation as $\beta$-*Cat* inactivation does (Glass et al., 2005). Third, neither $Lrp5^{+/-}$;$\beta$-$Cat_{gut}^{+/-}$ or $Tph1_{gut}^{+/-}$;$\beta$-$Cat_{gut}^{+/-}$ mice show the bone phenotype of $Lrp5^{-/-}$ or $Tph1_{gut}^{-/-}$ mice, respectively, indicating that Lrp5 and Tph1 on one hand and β-Cat on the other hand are not acting in the same pathways. It has been shown before that LiCl could correct the bone phenotype of the $Lrp5^{-/-}$ mice (Clement-Lacroix et al., 2005). This does not necessarily contradict this contention, because LiCl does not act exclusively on Wnt signaling and can affect many pathways in cells including serotonin in the brain, where its function, if any, in regulating bone mass is for now unknown (Basselin et al., 2005).

The homology of Arrow with Lrp5 extends to other Lrp proteins. Indeed, the closest Lrp5 relative, Lrp6, also has a high degree of homology with Arrow. This is important, because all genetic evidence obtained before the function of *Lrp5* during bone formation was discovered suggested that Lrp6 was a better candidate than Lrp5 to be a Wnt coreceptor. For instance, Lrp6 but not Lrp5 is required for Wnt signaling in *Xenopus* and in mice (Pinson et al., 2000; Tamai et al., 2004; Wehrli et al., 2000). Likewise, Wnt proteins fused to Frizzled could interact with Lrp6 but not with Lrp5 to activate a Wnt-responsive reporter gene (Holmen et al., 2002), *Lrp6* inactivation affects embryonic development whereas *Lrp5* inactivation does not (Pinson et al., 2000), and, more decisively, *Lrp6* inactivation affects bone resorption in a TCF-dependent manner like $\beta$-*Cat* osteoblast-specific inactivation does but does not impair bone formation as *Lrp5* loss-of-function mutation does (Glass et al., 2005; Kato et al., 2002; Kubota et al., 2008).

Our results do not rule out, however, that Lrp5, in other organs and/or in cell types other than osteoblasts, may transduce a Wnt signal. In particular, that the eye phenotype of the $Lrp5^{-/-}$ mice is not observed in the case of a duodenal- and osteoblast-specific deletion of *Lrp5* and is not corrected by decreasing extracellular serotonin concentration rules out a serotonin involvement in this abnormality. This observation provides indirect support to previous work showing an involvement of Lrp5 in Wnt signaling in that case (Lobov et al., 2005).

### Serotonin Signaling in Osteoblasts

Three serotonin receptors are expressed in osteoblasts. Two of them, *Htr2b* and *Htr2a*, are broadly expressed whereas *Htr1b* is, outside the brain, a fairly osteoblast-enriched gene. According to gene deletion experiments, Htr1b is the receptor responsible for the effect of serotonin on osteoblasts, whereas inactivation of either *Htr2b* or *Htr2a* does not affect bone mass. Our results regarding *Htr2b* are different from those of Collet et al. (2008). However, in their case, the bone phenotype was observed in mice lacking the gene in all cells, whereas in our case *Htr2b* was deleted only in osteoblasts. Thus, our results relate more tightly to the function (or lack) of this gene in osteoblasts. It is possible that the low bone mass phenotype they noted in the classical $Htr2b^{-/-}$ mice could be secondary to the decrease in heart function observed in these mice (Nebigil et al., 2000).

Until recently, the prevailing view of the transcriptional control of osteoblast biology is that it involves one of three players that are osteoblast specific: Runx2, Osx, and ATF4. Yet, none of these three transcription factors appears implicated in serotonin signaling in osteoblasts. Instead, CREB, a factor already known to affect osteoblast proliferation but that is broadly expressed (Fu et al., 2005), is the main transcriptional effector of serotonin signaling in osteoblasts. This observation, along with the emerging role in osteoblasts of other transcription factors such as, among others, Schnurri-3 or FOXO that are not osteoblast specific (Jones et al., 2006; Manolagas and Almeida, 2007), suggest that besides cell-specific transcription factors, other ones, more broadly expressed, will influence significantly differentiation and/or functions of the osteoblasts.

The demonstration that Lrp5 belongs to a novel endocrine axis regulating bone formation illustrates the power of an integrative approach to bone physiology. It also raises additional questions. For instance and from a biological point of view, we do not know yet how *Lrp5* expression affects *Tph1* expression in enterochromaffin cells of the gut. The structure of the Lrp5 protein raises the prospect that it may be a signaling receptor for a yet to be identified serotonin synthesis-inhibiting molecule. From a therapeutic point of view, the fact that ovariectomized $Tph1_{gut}^{-/-}$ mice do not display bone loss holds great promise given *Tph1* cell-specific expression. Further experiments will be needed to determine whether pharmacologically inhibiting Tph1 or use of a selective peripheral Htr1b receptor antagonist(s) is a valid approach in the treatment of postmenopausal osteoporosis.

## EXPERIMENTAL PROCEDURES

### Mice Generation

To generate osteoblast-specific and gut-specific gene-deficient mice for *Lrp5*, *Tph1*, and *Lrp5KI* mutations and *Htr2b* ($osb^{-/-}$, $gut^{-/-}$), targeting vectors harboring LoxP sites as well as a floxed neomycin resistance cassette were electroporated into embryonic stem cells (for details, see Figures S3–S5). ES cells containing the floxed allele (after $Neo^R$ removal) were injected in 129Sv/EV blastocysts to generate chimeric mice. *flox*/+ mice were crossed with $\alpha_1$*(I)Col-Cre* or *Villin-Cre* (obtained from the National Cancer Institute mouse repository) mice to generate $osb^{+/-}$ or $gut^{+/-}$ mice, and their progeny was intercrossed to obtain $osb^{-/-}$ or $gut^{-/-}$ mice. Generation of $Lrp5^{-/-}$,

*β-Cat*$^{fl/fl}$, *β-Cat(ex3)*$^{fl/fl}$, *Htr1b*$^{-/-}$, *Htr2a*$^{-/-}$, *Creb*$^{fl/fl}$, *Atf4*$^{-/-}$, *Runx2*$^{-/-}$, *Osx*$^{-/-}$, *Villin-Cre*, and *α*$_1$*(I)Col-Cre* mice was previously reported (Dacquin et al., 2002; el Marjou et al., 2004; Glass et al., 2005; Heath and Hen, 1995; Kato et al., 2002; Mantamadiotis et al., 2002; Weisstaub et al., 2006; Yang et al., 2004).

### Histology, Protein Expression, and Proliferation Assays

Immunohistochemistry was performed according to standard protocols on specimens embedded in paraffin and sectioned at 6 μm (Ducy et al., 2000). In vivo osteoblast proliferation assays (Zymed Laboratories) were performed on 4-week-old mice injected with BrdU (0.4 mg) and sacrificed 4 hr later. Static and dynamic histomorphometric analyses were performed on vertebral column specimens collected from 3-month-old mice using undecalcified sections according to standard protocols using the Osteomeasure analysis system (Osteometrics). Six to 12 animals were analyzed for each group.

### Cell Cultures and Bioassays

Primary osteoblasts were cultured as previously described (Ducy et al., 2000). Drug treatments were performed in 0.1% FBS. Cell proliferation was quantified by BrdU or MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays (Kato et al., 2002). Serum serotonin levels were quantified by ELISA (serotonin kit, Fitzgerald), whereas serotonin levels in brain regions were quantified by HPLC as described previously (Mann et al., 1992).

### Molecular Studies

RNA isolation and real-time PCR was performed following standard protocols. Genotypes of all the mice were determined by PCR (see Figures S3–S5). All primer sequences are available upon request.

### Statistical Analysis

Results are given as means ± standard deviations. Statistical analysis was performed by Student's t test. For Figures 1–7 and S1–S6, *$p < 0.05$ and **$p < 0.01$ versus WT or control.

## SUPPLEMENTAL DATA

Supplemental Data include 11 figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01255-5.

## ACKNOWLEDGMENTS

We thank Drs. J.P. Bilezikian, S. Cremers, D.A. Mackey, M. Zacharin, R. Lang, S. Rao, H. Schubert, and J. Tosi for patient samples and eye analysis; G. Ren and Yung-yu Huang for superb technical assistance; and Dr. M. Gershon for his critical reading of this manuscript. This work was supported by grants from the March of Dimes Foundation and the National Institutes of Health (to G.K.) and by the Shriners of North America (F.H.G.).

Received: May 27, 2008
Revised: August 12, 2008
Accepted: September 22, 2008
Published: November 26, 2008

## REFERENCES

Baron, R., and Rawadi, G. (2007). Targeting the Wnt/β-catenin pathway to regulate bone formation in the adult skeleton. Endocrinology *148*, 2635–2643.

Basselin, M., Chang, L., Seemann, R., Bell, J.M., and Rapoport, S.I. (2005). Chronic lithium administration to rats selectively modifies 5-HT2A/2C receptor-mediated brain signaling via arachidonic acid. Neuropsychopharmacology *30*, 461–472.

Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J., and Nusse, R. (1996). A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. Nature *382*, 225–230.

Boyden, L.M., Mao, J., Belsky, J., Mitzner, L., Farhi, A., Mitnick, M.A., Wu, D., Insogna, K., and Lifton, R.P. (2002). High bone density due to a mutation in LDL-receptor-related protein 5. N. Engl. J. Med. *346*, 1513–1521.

Clement-Lacroix, P., Ai, M., Morvan, F., Roman-Roman, S., Vayssiere, B., Belleville, C., Estrera, K., Warman, M.L., Baron, R., and Rawadi, G. (2005). Lrp5-independent activation of Wnt signaling by lithium chloride increases bone formation and bone mass in mice. Proc. Natl. Acad. Sci. USA *102*, 17406–17411.

Collet, C., Schiltz, C., Geoffroy, V., Maroteaux, L., Launay, J.M., and de Vernejoul, M.C. (2008). The serotonin 5-HT2B receptor controls bone mass via osteoblast recruitment and proliferation. FASEB J. *22*, 418–427.

Dacquin, R., Starbuck, M., Schinke, T., and Karsenty, G. (2002). Mouse α1(I)-collagen promoter is the best known promoter to drive efficient Cre recombinase expression in osteoblast. Dev. Dyn. *224*, 245–251.

Ducy, P., Amling, M., Takeda, S., Priemel, M., Schilling, A.F., Beil, F.T., Shen, J., Vinson, C., Rueger, J.M., and Karsenty, G. (2000). Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. Cell *100*, 197–207.

el Marjou, F., Janssen, K.P., Chang, B.H., Li, M., Hindie, V., Chan, L., Louvard, D., Chambon, P., Metzger, D., and Robine, S. (2004). Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis *39*, 186–193.

Fu, L., Patel, M.S., Bradley, A., Wagner, E.F., and Karsenty, G. (2005). The molecular clock mediates leptin-regulated bone formation. Cell *122*, 803–815.

Gershon, M.D., and Tack, J. (2007). The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology *132*, 397–414.

Glass, D.A., II, Bialek, P., Ahn, J.D., Starbuck, M., Patel, M.S., Clevers, H., Taketo, M.M., Long, F., McMahon, A.P., Lang, R.A., et al. (2005). Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. Dev. Cell *8*, 751–764.

Gong, Y., Slee, R.B., Fukai, N., Rawadi, G., Roman-Roman, S., Reginato, A.M., Wang, H., Cundy, T., Glorieux, F.H., Lev, D., et al. (2001). LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell *107*, 513–523.

Heath, M.J., and Hen, R. (1995). Serotonin receptors. Genetic insights into serotonin function. Curr. Biol. *5*, 997–999.

Hediger, M.L., England, L.J., Molloy, C.A., Yu, K.F., Manning-Courtney, P., and Mills, J.L. (2008). Reduced bone cortical thickness in boys with autism or autism spectrum disorder. J. Autism Dev. Disord. *38*, 848–856.

Holmen, S.L., Salic, A., Zylstra, C.R., Kirschner, M.W., and Williams, B.O. (2002). A novel set of Wnt-Frizzled fusion proteins identifies receptor components that activate β-catenin-dependent signaling. J. Biol. Chem. *277*, 34727–34735.

Jones, D.C., Wein, M.N., Oukka, M., Hofstaetter, J.G., Glimcher, M.J., and Glimcher, L.H. (2006). Regulation of adult bone mass by the zinc finger adapter protein Schnurri-3. Science *312*, 1223–1227.

Kato, M., Patel, M.S., Levasseur, R., Lobov, I., Chang, B.H., Glass, D.A., II, Hartmann, C., Li, L., Hwang, T.H., Brayton, C.F., et al. (2002). Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. J. Cell Biol. *157*, 303–314.

Krishnan, V., Bryant, H.U., and MacDougald, O.A. (2006). Regulation of bone mass by Wnt signaling. J. Clin. Invest. *116*, 1202–1209.

Kubera, M., Kenis, G., Bosmans, E., Scharpe, S., and Maes, M. (2000). Effects of serotonin and serotonergic agonists and antagonists on the production of interferon-γ and interleukin-10. Neuropsychopharmacology *23*, 89–98.

Kubota, T., Michigami, T., Sakaguchi, N., Kokubu, C., Suzuki, A., Namba, N., Sakai, N., Nakajima, S., Imai, K., and Ozono, K. (2008). Lrp6 hypomorphic mutation affects bone mass through bone resorption in mice and impairs interaction with Mesd. J. Bone Miner. Res. *23*, 1661–1671.

Lobov, I.B., Rao, S., Carroll, T.J., Vallance, J.E., Ito, M., Ondr, J.K., Kurup, S., Glass, D.A., Patel, M.S., Shu, W., et al. (2005). WNT7b mediates macrophage-induced programmed cell death in patterning of the vasculature. Nature *437*, 417–421.

Mann, J.J., McBride, P.A., Brown, R.P., Linnoila, M., Leon, A.C., DeMeo, M., Mieczkowski, T., Myers, J.E., and Stanley, M. (1992). Relationship between central and peripheral serotonin indexes in depressed and suicidal psychiatric inpatients. Arch. Gen. Psychiatry *49*, 442–446.

Manolagas, S.C., and Almeida, M. (2007). Gone with the Wnts: β-catenin, T-cell factor, forkhead box O, and oxidative stress in age-dependent diseases of bone, lipid, and glucose metabolism. Mol. Endocrinol. *21*, 2605–2614.

Mantamadiotis, T., Lemberger, T., Bleckmann, S.C., Kern, H., Kretz, O., Martin Villalba, A., Tronche, F., Kellendonk, C., Gau, D., Kapfhammer, J., et al. (2002). Disruption of CREB function in brain leads to neurodegeneration. Nat. Genet. *31*, 47–54.

Moon, R.T., Kohn, A.D., De Ferrari, G.V., and Kaykas, A. (2004). WNT and β-catenin signalling: diseases and therapies. Nat. Rev. Genet. *5*, 691–701.

Nebigil, C.G., Choi, D.S., Dierich, A., Hickel, P., Le Meur, M., Messaddeq, N., Launay, J.M., and Maroteaux, L. (2000). Serotonin 2B receptor is required for heart development. Proc. Natl. Acad. Sci. USA *97*, 9508–9513.

Pinson, K.I., Brennan, J., Monkley, S., Avery, B.J., and Skarnes, W.C. (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. Nature *407*, 535–538.

Rand, M., and Reid, G. (1951). Source of 'serotonin' in serum. Nature *168*, 385.

Richards, J.B., Papaioannou, A., Adachi, J.D., Joseph, L., Whitson, H.E., Prior, J.C., and Goltzman, D. (2007). Effect of selective serotonin reuptake inhibitors on the risk of fracture. Arch. Intern. Med. *167*, 188–194.

Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J.P., and He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. Nature *407*, 530–535.

Tamai, K., Zeng, X., Liu, C., Zhang, X., Harada, Y., Chang, Z., and He, X. (2004). A mechanism for Wnt coreceptor activation. Mol. Cell *13*, 149–156.

Toomes, C., Bottomley, H.M., Jackson, R.M., Towns, K.V., Scott, S., Mackey, D.A., Craig, J.E., Jiang, L., Yang, Z., Trembath, R., et al. (2004). Mutations in LRP5 or FZD4 underlie the common familial exudative vitreoretinopathy locus on chromosome 11q. Am. J. Hum. Genet. *74*, 721–730.

Warden, S.J., Robling, A.G., Sanders, M.S., Bliziotes, M.M., and Turner, C.H. (2005). Inhibition of the serotonin (5-hydroxytryptamine) transporter reduces bone accrual during growth. Endocrinology *146*, 685–693.

Wehrli, M., Dougan, S.T., Caldwell, K., O'Keefe, L., Schwartz, S., and Vaizel-Ohayon, D. (2000). Arrow encodes an LDL-receptor-related protein essential for Wingless signalling. Nature *407*, 527–530.

Weisstaub, N.V., Zhou, M., Lira, A., Lambe, E., Gonzalez-Maeso, J., Hornung, J.P., Sibille, E., Underwood, M., Itohara, S., Dauer, W.T., et al. (2006). Cortical 5-HT2A receptor signaling modulates anxiety-like behaviors in mice. Science *313*, 536–540.

Yang, X., Matsuda, K., Bialek, P., Jacquot, S., Masuoka, H.C., Schinke, T., Li, L., Brancorsini, S., Sassone-Corsi, P., Townes, T.M., et al. (2004). ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology. Cell *117*, 387–398.

Zaidi, M. (2007). Skeletal remodeling in health and disease. Nat. Med. *13*, 791–801.